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Determination by LC–MS/MS of Colistins A and B in Plasma and Ultrafiltrate From Critically Ill Patients Undergoing Continuous Venovenous Hemodiafiltration

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Background: Colistin is a 50-year-old antibiotic, the use of which was ceased in the 70s and recently resumed as a “salvage therapy” against multidrug-resistant gram-negative bacteria, such as Pseudomonas aeruginosa and Acinetobacter baumannii. The narrow therapeutic range of colistin makes the choice of its correct dosage crucial, and monitoring of blood concentration is occasionally necessary for critically ill patients, including intensive care patients subjected to continuous renal replacement therapy. **Methods:** Two LC–MS/MS methods were developed and fully validated for the quantitative determination of colistins A and B in plasma and dialysis ultrafiltrate (UF) samples, ultimately arising from 4 patients undergoing continuous venovenous hemodiafiltration (CVVHDF). **Results:** The developed methods proved to be both specific and selective. They showed good fit and linearity over the entire range of interest. Trueness and accuracy proved satisfactory. Both methods have excellent intraassay precision (percent coefficient of variations were lower than 10%) and limit of detection values in the range 20–100 ng/mL, about 1–2 orders of magnitude below the concentrations commonly detected in real samples. The mean sieving coefficient (SC) values, measured after 10 minutes of CVVHDF, were 0.42 for colistin A and 0.48 for colistin B. SC values proved to be quite stable for 24 hours, but then declined to 0.24 for colistin A and 0.32 for colistin B, respectively, after 48 hours. At the median blood flow and effluent flow rate of 120 and 28 mL/min, clearance values for colistin B were higher than 15 mL/min. During the entire duration of CVVHDF sessions, the SC and clearance values for colistin A were significantly lower than colistin B. **Conclusions:** Two simple methods for the simultaneous determination of colistins A and B have been developed and validated. Their application in the clinical setting demonstrates that CVVHDF treatment lasting 48 hours produces a relatively constant and efficient removal of the drug.

Key Words: colistin, LC–MS/MS, dialysis, CVVHDF, ultrafiltrate

INTRODUCTION

Polymyxins include 5 groups of polypeptidic antibiotics discovered in the 1950s,1 among which only polymyxin B and polymyxin E are employed in clinical therapy. Polymyxin E, also called colistin, is produced by Bacillus colistinus. This polypeptide mixture is constituted by D and L amino acids, such as D-leucine, L-threonine, and L-a-g-diaminobutyric acid. They are arranged to form cyclic heptapeptides, with a tripeptidic lateral chain linked to a fatty acid by covalent a-amidic bond. Colistin included more than 30 components, distinguished by amino acid and fatty acid residue composition. The 2 major components are colistin A (polymyxin E1) and colistin B (polymyxin E2). The abundance ratio between these 2 colistins in both commercial materials and analytical standards is variable and depends on the suppliers and batches.2

Colistin is used for the treatment of infections caused by gram-negative bacteria. It presents hydrophobic and basic properties, respectively, because of the fatty acid residue and 5 free amino groups. Colistin interacts with the anionic groups of the lipopolysaccharides (LPS) contained in the membrane of gram-negative bacteria, displacing Mg^{2+} and Ca^{2+} cations from the negatively charged phosphate groups of the LPS lipid A. This causes an increased permeability of the membrane, resulting in the cellular death.3

Colistin methanesulphonate (CMS) is a prodrug of colistin, obtained by the reaction of the free amino residues of colistin with formaldehyde and sodium bisulfite. It is significantly less toxic than colistin. Both CMS and colistin sulphate are scarcely adsorbed by the gastrointestinal tract, with the exception of the cases of newborns and patients with gastric mucosa alteration. Therefore, colistin is principally administered as CMS by intramuscular or intravenous injection. CMS is then hydrolyzed to colistin and other intermediate species.4,5

The use of colistin as an antibiotic agent was ceased in the 70s because of its nephrotoxicity and neurotoxicity and was substituted by aminoglycosides. In particular, nephrotoxicity was described as the main risk associated with the prolonged administration of colistin.6,7 However, recent studies concluded that the nephrotoxicity of colistin is less severe than it was reported in the past. In particular, the risk of CMS nephrotoxicity was found to be significant only after 14 days of administration.8 Because the risk seems to be linked to its cumulative dosage, not to single administration, it stems the need to monitor the renal function of patients subjected to prolonged colistin therapy. These differences between old and recent toxicological data are because of the improvement of medical care, renal function monitoring, and careful selection of coadministered drugs. In the late 90s, the use of colistin was resumed as a “salvage” therapy against multidrug-resistant gram-negative bacteria, such as Pseudomonas aeruginosa, Acinetobacter baumannii, and Klebsiella pneumoniae,9 because new resistant bacteria strands could not be eradicated by traditional or last-generation antibiotics.

The pharmacokinetic and pharmacodynamic data about CMS and colistin are relatively scarce, since 1959, when they entered in clinical use, the strict drug development

procedures that are now mandatory were not required yet. It follows that dosage regimens are rarely described for specific categories of patients, in particular for critically ill patients with altered renal function and subjected to renal replacement therapies.3

Recommended CMS dosage during hemodialysis is 80 mg after each cycle of dialysis,10 but the clearance conditions observed in this case are different from those of patients subjected to continuous renal replacement therapy (CRRT) when clear pharmacological indication is missing.11 A few studies showed that the pharmacokinetics of CMS/colistin is altered in patients under critical conditions5 or affected by cystic fibrosis.12 The colistin clearance is essentially not renal, and no correlation was found between colistin and creatinine clearance.13 However, creatinine clearance significantly affects the total body clearance of CMS and colistin.14

The first pharmacokinetics of colistin was obtained by microbiological experiments.15 Afterwards, analytical methods based on immunological assays, thin layer chromatography, capillary electrophoresis, and high-pressure liquid chromatography (HPLC) were developed. More recently, LC–MS/MS methods have been developed for the determination of polymyxin B1 and B2,16 colistin, and CMS in plasma and other biological samples,17,18 including UF.19 No reference methods exist for separate quantitative determination of colistins A and B, but rather a few laboratories developed and validated in-house methods, addressed to the measurement of polymyxin and colistin in human plasma.16,18 After considering the existing methods, we developed and validated 2 LC–MS/MS methods devoted to the quantitative determination of colistins A and B in plasma and UF, respectively, using polymyxin B as the internal standard (IS). In addition, our analytical protocols were applied to monitor colistin concentrations in plasma and UF arising from critically ill patients undergoing CRRT. This allowed us to study colistin extracorporeal clearance and its removal efficiency over the dialysis session.

MATERIALS AND METHODS

Chemicals

Colistin sulphate, polymyxin B sulphate, acetonitrile, formic acid, and methanol were purchased from Sigma Aldrich (Milan, Italy). Acetone was from Carlo Erba (Milan, Italy). Sep-Pac Vac 3 cc (200 mg) C18 solid phase extraction cartridges were obtained from Waters (Milan, Italy). Highly purified water was produced by Millipore Milli-Q UF Plus system (Millipore, Bedford, MA).

Plasma and UF Samples

Human blank plasma was obtained from healthy volunteers. The solution for hemodiafiltration CB 32-HDF B200 was purchased from NovaSelect S.p.A (Potenza, Italy). Ex vivo samples were obtained from critically ill patients undergoing CRRT at the Intensive Care Department, CTO Hospital (Ospedale CTO), Turin (see Patients). To avoid degradation of CMS during sampling, blood samples were directly chilled and thereafter

centrifuged as soon as possible. Plasma samples were then stored at 280°C until analysis.20

Stock and Working Standard Solutions

Stock standard solutions of colistin and polymyxin B (IS) were prepared in water with 0.1% formic acid, at a concentration of 1000 mcg/mL. Working standard solutions were obtained by appropriate dilutions. Stock and working standard solutions were stored at 2208C in the dark.

Sample Preparation for Colistin Determination in Plasma

The samples were quickly thawed in cold water in batches of 12 samples or less. Hundred microliters of human plasma was transferred into a 10-mL glass tube. Twenty microliters of the IS working solution (10 ng/mL) and 200 mL of acetone were added in sequence. The sealed tube was shaken vigorously for 3 minutes by means of a vortex multimixer (Tecnovetro, Monza, Italy) and then centrifuged at g value of 1459 m/s2 for 5 minutes (model Megafuge 1.0 Heraeus; ASHI, Milan, Italy). Two hundred microliters of the supernatant was transferred into a new 10-mL glass tube and evaporated to dryness under a gentle stream of nitrogen and mild heating (408C), using a Techne Sample Concentrator (Barloworld Scientific, Stone, UK). The residue was redissolved in 100 mL of 0.1% HCOOH/CH3CN (95:5, vol/vol) solution and transferred into the analytical vial for the HPLC–MS/MS analysis. Because worked-up samples were verified to be stable at low pH values for 5 hours at least,20 their instrumental analysis was always planned within 2 hours from the end of the preparation step.

Sample Preparation for Colistin Determination in UF

The samples were quickly thawed in cold water in batches 12 samples or less. A 50 mL aliquot of UF real sample was transferred into a 10-mL glass tube and diluted with 50 mL of solution for hemodiafiltration. Twenty microliters of the IS working solution (10 ng/mL) and 50 mL of drug-free human plasma were added to the same glass tube. Then, the sample volume was brought to 1 mL by adding highly purified water and was briefly vortex-mixed. The mixture was loaded onto a solid phase extraction column, preconditioned with 1 mL of methanol followed by 1 mL of water. The column was subsequently washed with 1 mL of water, and the analytes were eluted with 1 mL of 0.1% (vol/vol) formic acid in methanol. Then, the eluate was evaporated to dryness under a gentle stream of nitrogen at 408C, and the residue was redissolved in 100 mL of 0.1% HCOOH/CH3CN (95:5, vol/vol) solution and transferred into the analytical vials for the LC–MS/MS analysis. As for plasma samples, the instrumental analysis was always executed within 2 hours after the preparation step.

LC–MS/MS Procedure

The chromatographic separation was performed by an Agilent 1100 series liquid chromatograph (Agilent Technologies, Palo Alto, CA), including a vacuum degasser, a binary pump, an autosampler, and a column thermostat. The liquid chromatograph was equipped with a Phenomenex Synergi Fusion-RP C18 80 Å (4 mm) 150 · 2.00 mm column and a Phenomenex SecurityGuard 4.0 · 2.0 mm precolumn. The chromatographic run was carried out using a binary mobile phase of 0.1% HCOOH in highly purified water (A) and 0.1% HCOOH in acetonitrile (B), under the following program: linear gradient from 95% to 50% acetonitrile in 2 minutes, linear gradient from 50% to 95% acetonitrile in 1 minute, isocratic with 95% acetonitrile for 8 minutes, total run time: 11 minutes. The injection volume was 20 mL, and the flow rate was 0.3 mL/min. The LC was interfaced to an Applied Biosystems API 3200 triple quadrupole mass spectrometer (Applied Biosystems Sciex, Ontario, Canada), operating in the electrospray ionization (ESI)—positive ion mode. The other MS parameters were set as follows: curtain gas, 25 psi; collision gas, 10 psi; ion spray voltage, 2500 V; probe temperature, 5008C; ion source gas-1, 50 psi; ion source gas-2, 40 psi; entrance potential, 10 V; collision cell exit potential, 10 V. Ion acquisition was operated at unit mass resolution in the selected reaction monitoring (SRM) mode, using the doubly charged molecular ion [MH2]2+ as the precursor ion, both for colistin and IS. The doubly charged ions are more intense than the singly charged ions, as already reported by Sin et al (Sin et al, 2005). The SRM ion transitions considered, together with the relative values for declustering potential and collision energy, are reported in Table 1.

Methods Validation

All the validation parameters generally required for quantitative bioanalytical procedures21 were determined, including selectivity, linearity, stability, inaccuracy, imprecision, lower limit of quantification (LLOQ), limit of detection (LOD), and recovery. Moreover, validation parameters typical for LC–ESI-MS methods were assessed, including matrix effects (ME) that is, ESI ion suppression or ion enhancement, and carryover.

Specificity

Ten samples of blank plasma and 10 aliquots of blank UF were analyzed. The occurrence of possible interferences from endogenous substances was tested by monitoring the SRM chromatograms characteristic for both analytes at the expected retention time interval.

Selectivity

The selectivity was evaluated at 2 concentrations. Twenty samples of negative plasma and 20 aliquots of UF were spiked with colistins A and B and then analyzed. The 2 spiking levels for colistins A and B in plasma samples were respectively 305 and 618 ng/mL (low level) and respectively 3050 and 6180 ng/mL (high level). The 2 spiking concentrations for

colistins A and B in UF samples were 152.5 and 309 ng/mL (low level) and 1525 and 3090 ng/mL (high level), respectively.

Linearity

Calibration curves for colistins A and B in plasma and UF matrices were built by spiking blank real samples with the appropriate concentrations of the analytes. Each curve included 6 concentration levels (including zero level), over a range covering the concentrations expected in the real samples obtained from patients. The investigated ranges were 0–9150 ng/mL for colistin A (0, 152.5, 305.0, 1525, 3050, and 9150 ng/mL) and 0–18,540 ng/mL for colistin B (0, 309, 618, 3090, 6180, and 18,540 ng/mL) in plasma matrix and 0–3050 ng/mL for colistin A (0, 91.5, 152.5, 305.0, 1525, and 3050 ng/mL) and 0–6180 ng/mL for colistin B (0, 185.4, 309, 618, 3090, and 6180 ng/mL) in UF matrix. Each level was determined in triplicate for the plasma matrix and in duplicate for the UF matrix.

Inaccuracy and Trueness

The inaccuracy is expressed as the percent deviation from the accepted reference value and is used to evaluate the systematic error of the method.21 The inaccuracy was calculated by comparing the concentrations determined in the spiked samples used for the specificity tests with their nominal concentration. Both within-run and between-run inaccuracy were determined.

Imprecision

The imprecision is generally expressed as an absolute or relative standard deviation from the mean of repeated determinations. We evaluated the repeatability of the method, representing the imprecision under the same operating conditions, over a short interval of time, usually defined as within-run imprecision.21 We evaluated also the repeatability of the method over a longer interval of time, usually defined as between-run imprecision. Both within-run and between run imprecision were determined at 2 concentration levels, as percent coefficient of variation (CV%) from the mean values.

Limit of Detection

LOD is the lowest concentration of the analyte in a real sample, for which specific identification criteria can still be fulfilled.21 LODs were calculated from the signal-to-noise (S/N) values obtained from the chromatographic profiles of the SRM transition with the lowest intensity among the SRM transitions characterizing each analyte. S/N values were extrapolated from the 3 lowest levels of the calibration curves. Calculated LOD values were experimentally confirmed by analyzing further spiked samples at concentrations slightly higher than the calculated LODs.

Lower Limit of Quantification

The LLOQ is the lowest amount of the analyte in a real sample that may be quantitatively determined with suitable precision and accuracy.21 LLOD values were calculated as 3 times the LOD values.

Stability

The stability of standard solutions had been carefully evaluated by Orwa et al.22 Their results indicate that colistin and polymyxin B are susceptible to degradation in both neutral and basic condition (pH . 5). Accordingly, our stock and working solutions were prepared in HCOOH 0.1%,22 and were periodically tested for stability throughout the study.

ME and Recovery

Suppression or enhancement of the ionization of the analytes by the effect of coeluting compounds is a well known phenomenon occurring in LC–MS analysis, especially when ESI is used. Recovery represents the percentage of the analyte still present after sample workup, as compared with the amount of the analyte initially contained in the sample. ME and recovery were determined within the same analytical session by preparing 3 sets of samples, each including 5 replicates. In the first set, blank plasma and UF samples were spiked with the analytes before the extraction step; in the second set, working solutions of the analytes were added (at the same concentrations) on the blank plasma and UF extracts; the third set was represented by the working solutions at the same concentrations. The recovery of the analytes was calculated by the ratio between the analyte concentration determined after its extraction (first set) and that determined on the spiked extract (second set). The ME was calculated as the percentage ratio between the analyte chromatographic peak area detected from the second set and that detected from the third set. The difference with respect to 100% highlighted matrix suppression (values below 100%) or enhancement (values above 100%).21 ME was determined also for the ISs, at the concentration used in analysis of real samples. IS-normalized ME have been calculated23 (Table 2).

Carryover

Carryover effect was evaluated by injecting an alternate sequence of blank plasma (and UF) samples and samples spiked with the analytes at the highest concentration of the calibration curves (plasma spiked at 9150 ng/mL for colistin A and 18,540 ng/mL for colistin B; and UF spiked at 3050 ng/mL for colistin A and 6180 ng/mL for colistin B). Carryover effects were positively detected when the S/N values for SRM chromatographic profiles exceeded 3, in the analysis of blank samples.

Ex Vivo Study
Patients

Four serial critically ill patients, admitted to the Intensive Care Department at CTO Hospital, with severe sepsis/septic shock sustained by infection of multiresistant gram-negative bacteria were included in this study (Table 3). All these patients showed residual sensibility to colistin, suffered from acute kidney injury, and underwent continuous venovenous hemodiafiltration (CVVHDF) with regional citrate anticoagulation. The study protocol was in accordance with the Helsinki Declaration. Informed written consent was obtained by either a close relative or a legal representative.

CVVHDF Protocol

CVVHDF procedures were standardized and carried out with a Multi-filtrate apparatus (Fresenius Medical Care AG, Bad Homburg, Germany) equipped with high-flow polysulfone filter (AV1000, surface area of 1.8 m2; Fresenius Medical Care) and setting replacement fluid infusion in predilution. The blood circuit was treated with citrate (ACD-A [Anticoagulant Citrate Dextrose Solution A]-containing replacement solution infused in predilution; citrate concentration, 28.8 mmol/L) to prevent coagulation and to reduce the risk of hemorrhagic complications.24 Calcium replacement infusion was prepared by using a commercial 10% calcium chloride solution infused in a separate line at the end of the venous circuit.25 The CVVHDF blood flow rate was set at 120 mL/min and the predilution infusion rate at 900 mL/h to reach a prefilter serum citrate of 3.3 mmoL/L. Administration of calcium chloride 10% solution started at a flow rate of 4.0 mL/h, followed by 0.5 mL/h increments for each effluent increase of 500 mL.26 Dialysate flow rate was started at 800 mL/h, and was modified according to the patients' metabolic and fluid balance requirements. As dialysate, calciumfree fluid-containing bags (CiCa bag; Fresenius Medical Care) were used.

Colistin Methanesulphonate Infusion and Sampling Protocol

Colistin methanesulphonate (Colimicina; UCB Pharma SpA, Pianezza, Italy) was administered at the dose of 4.5 · 106 units (equivalent to 360 mg of CMS, or 135 mg of colistin base equivalent27) in 250 mL of normal saline solution every 12 hours by intravenous infusion for more than 60 minutes. Because of the lack of clear recommendations based on clinical evidence, CMS dose typically prescribed was not modified for patients undergoing CRRT.5 In 1 patient (patient 4), the administered dose was 3.0 · 106 units (equivalent to 240 mg of CMS) every 12 hours intravenously of more than 60 minutes. All samples were taken from patients after the third day of therapy with colistin that is at the pharmacokinetic steady state condition. Samples were drawn respectively at 10 minutes, 3, 24, and 48 hours after the beginning of the dialysis session (Table 4). Further samples were collected at 6 and 12 hours, whenever possible.
Blood samples were drawn directly from systemic arterial blood for measuring plasma concentration of colistin entering the filter (Cpl-in) and from the circuit venous line (between the filter and the venous air bubble trap) for measuring plasma concentration of colistin leaving the filter (Cpl-out). Ultrafiltrate samples were taken from the ultrafiltrate line (C-UF). Plasma and effluent samples were stored at 2208C and were analyzed within a week. Colistin extraction depends on its sieving coefficient (SC), that is, the concentration in ultrafiltrate (C-UF) divided by the mean of concentrations in pre- and post-filter blood [(Cpl-in + Cpl-out)/2]. Calculated filter clearance of colistin A (Clear Filter, expressed as mL/min), is given by the product of SC and the ultrafiltration rate (eff flow), expressed as effluent flow (mL/min).

Statistical Methods

The software program Statistica (Statistica 6.1; Stat-Soft Inc, Tulsa, OK) was used for descriptive statistics and graphs. All values were expressed as median (interquartiles range).

RESULTS

Validation of the Analytical Methods

Figure 1 shows typical SRM chromatographic profiles for colistin A, colistin B, and the ISs, polymyxin B1, and B2. Although the chromatographic peaks seem to be overlapped, all SRM transitions for the 4 compounds are different, resulting in separate acquisition channels and yielding no interferences among analytes and IS, as is evident in Figure 1. The LC elution program needed an initial pulse of high acetonitrile content to control the chromatographic peak width, followed by a regular gradient, as is described in a previous study.28 A summary of validation results is reported in Table 2.

Specificity and Selectivity

The virtual absence of any interfering signal (S/N , 3) in the SRM chromatograms at the retention times of the analytes of interest was verified for all blank samples, demonstrating that the method is specific for the tested compounds and free from positive interference from other plasma and UF components. The analytes were clearly identified in all the spiked samples, according to the criteria reported in the WADA Technical Document—TD2010IDCR,29 that were fully satisfied.

Linearity

The calibration curves obtained for colistins A and B in both plasma and UF showed good fit and linearity over the entire range of interest. The resulting R2 values ranged from 0.9970 to 0.9995.

Inaccuracy and Trueness

For both methods, experimental trueness values proved satisfactory, yielding percent deviations lower than 615%. The same conclusion applies to inaccuracy evaluation; only for colistin A at the low spiking level in plasma, a few single determinations exhibited biases slightly higher than 15%, but always within the range of 620%.

Imprecision

All experimental CV% were lower than 10% for both the methods (Table 2), proving that the methods have excellent within-run and between-run precisions.

LOD and LLOQ

All LOD values reported in Table 2 are in the range 20–100 ng/mL, about 1–2 orders of magnitude below the concentrations commonly detected in the real plasma and UF samples. Also LLOQ concentration values are significantly lower than those recorded in real samples, demonstrating that the methods are suitable to provide unequivocal detection and accurate quantitative determination of the analytes.

Stability

Using the storage conditions described in the previous section, stock and working solutions proved to be stable for the entire period of the present study. Fresh working solution was periodically prepared.

ME and Recovery

Homogeneous and significant signal suppression because of the matrix was observed for colistins A and B, and polymyxins B1 and B2, as expected for structurally similar and partially coeluting substances. In plasma samples, ME ranges from 27% to 39% for analytes and IS at both low and high validation levels, with slightly lower values (higher ME) at high concentration. Similar percentages were observed in UF samples, again with slightly higher effects recorded at high concentrations. The ME homogeneity and stability between analytes and IS provide similar signal suppression and a compensating outcome, resulting in accurate and repeatable quantitative determinations, as described above. The ISnormalized ME was calculated at low and high concentration levels and the observed CV% proved not to be larger than 15%, as prescribed by EMA (European Medicines Agency) Guidelines.23 Recoveries were also uniform for all colistins and polymyxins, ranging from 46% to 62% for plasma samples, and from 66% to 84% for UF samples. The addition of blank human plasma to the UF in the sample preparation was necessary to obtain good extraction efficiency from UF samples. The incomplete recovery of analytes and IS from both matrices has to be attributed to a partial coprecipitation of these polypeptides with the blood proteins, produced by acetone addition during the sample treatment.

Carryover

Both methods proved to be affected by carryover effect. This is likely to be caused by colistin adsorption on various materials, as reported by Gobin et al4 and by Jansson et al.20 To avoid contamination between consecutive samples, 1 injection and subsequent chromatographic run of pure solvent (acetonitrile) into the LC–MS/MS instrument was executed after each sample. The virtual absence of further carryover effect using this procedure was positively verified (Fig. 1).

Ex Vivo Concentrations Measurement and Clearances of Colistin in CVVHDF Patients

The experimental protocol envisaged that samples were collected after 10 minutes, 3 hours, 24 hours, and 48 hours, at least, after the beginning of the CVVHDF session. Problems encountered during the intensive care for these critical patients resulted in occasional impracticality to collect the samples at the planned time. For 1 patient, additional sampling after 6 and 12 hours was done (Table 4). The absolute concentrations measured in blood samples depend on several parameters, including dosage, time of administration, body-mass index, and others. For our aim, much more interesting is the ratio between UF and blood concentration, that is, the SC, which illustrates the colistin distribution in the 2 phases. To make this information clearer, the SC values have been averaged for 4 patients, whenever possible. The outcome is reported in Figure 2. After 10 minutes of CVVHDF, SC values were 0.42 for colistin A and 0.48 for colistin B (Fig. 2).

Apart from colistin B, whose SC peaked to 0.58 after 3 hours, SC values proved to be quite stable for 24 hours, but declined to 0.24 for colistin A and 0.32 for colistin B after 48 hours (Fig. 2). During the entire duration of the of CVVHD sessions, the SC values for colistin A were significantly lower than those for colistin B. Figure 3 shows the clearance values for colistins A and B. The highest clearance values (above 15 mL/min) were found after 3 and 6 hours for colistin B at the median blood flow and effluent flow rate of 120 and 28 mL/min, respectively. The global trends depicted in Figure 3 closely resemble the SC pattern depicted in Figure 2, showing reduced clearance values after 48 hours and lower clearance values measured for colistin A than for colistin B. In particular, clearance values declined for all patients during the CVVHDF session, reaching half the starting value after 48 hours of treatment.

DISCUSSION

Development of the Methods

Colistins A and B are not commercially available as separated pure reference standards, but are sold as mixtures. To evaluate the relative abundance of the 2 components, it was assumed that colistins A and B had the same response factors for the corresponding SRM transitions. This assumption is reasonable, because the 2 molecular structures differ only for a single CH2 group. The percentage of each component was determined by the peak area for its main SRM transition with respect to the sum of peak areas for the 2 components.17 Polymyxin B was used as the IS. Like colistin, polymyxin is a mixture of various polypeptidic compounds with similar molecular structures, but the main components are polymyxin B1 and polymyxin B2. Polymyxin B1 is generally chosen as the IS for both colistin A and colistin B. In the course of our validation experiments, we noticed that the use of polymyxin B1 as the IS in UF samples produced a moderate overestimation of colistin B, whereas it was optimal for the determinations of both colistin homologues in plasma and for colistin A in UF samples (see IS-normalized ME in Table 2). Similar phenomenon has been reported by Thomas et al16; they developed a LC–MS/MS method for polymyxin B1 and polymyxin B2, with colistin as the IS, where they observed significant ionization enhancement at low polymyxin concentrations when colistin was present. This effect was observed to become less pronounced at higher polymyxin B1 and B2 concentrations. Their analytical strategy led them not to use any internal standardization.16 In our case, more accurate quantification was obtained by averaging the signals of polymyxin B1 and polymyxin B2 to yield more closely
matching results.

Results in Patients Undergoing CVVHDF

Colistin monitoring is occasionally needed in patients undergoing dialysis therapy, in that case, the renal clearance is impaired, and a fraction of the drug is eliminated through the ultrafiltrate effluent. Renal function, expressed as creatinine clearance, is the main pharmacokinetic factor involved in the maintenance dose of colistin methanesulphonate.14 Out of an estimated colistin total body clearance of 45.3 mL/min (the sum of renal clearance and non-renal-dependent component), 29.4 mL/min is the estimated volume because of renal clearance. 14 When the renal function is severely impaired and the patient undergoes renal replacement therapy, the renal component of total colistin clearance is virtually absent and has to be substituted by filter clearance. The

extracorporeal clearance of colistin depends on its elimination through the polysulfone dialysis membrane, characterized by an anionic surface without adsorptive properties. This polysulfone material forms a membrane allowing high flow and is able to remove substances with molecular weight up to 30 kDa.30 Because colistin has a molecular weight slightly exceeding 1 kDa, it freely crosses this polysulfone membrane, leaving a reduced concentration in the blood stream after the dialysis. The whole fraction of colistin eliminated by the CVVHDF procedure is supposed to be found in the effluent. The colistin SC value depends on the properties of both the membrane and solute and is experimentally determined to measure the removal efficiency of the filter. For example, progressive filter clogging is evidenced by a decay of the SC. Figure 2 evidences that the initial SC for colistin A and B (0.42 and 0.48, respectively) declined to about 0.24 and 0.32 after 48 hours of continuous use. Even though colistin is mostly bound to albumin in plasma, the free colistin fraction in the blood stream has been recently evaluated as 34%.31 Colistin A is likely to be more extensively bound to albumin than colistin B, because of its longer fatty acid residue,31 which explains its lower SC. In fact, only the free plasmatic fractions of colistins A and B can cross the dialysis membrane and reach the effluent. Besides this difference, the SC values for colistins A and B are relatively high, with a mean SC of 45% at the beginning of treatment (Fig. 2) and the removal efficiency of the system is still relatively high at the end of treatment, when the membrane efficiency is known to be affected by extensive fouling processes.32 From Figure 3, it is possible to evaluate the efficiency of colistin removal during a therapeutic session. In the early hours of CRRT at a blood flow of 120 mL/min and effluent rate of 2 L/h, the clearances of colistins A and B were at about 11 and 15 mL/min, respectively; then, they slowly declined along the treatment. From these data, the total colistin removal from the blood stream can be calculated from mean clearance values of 9.4 and 12.8 mL/min for colistins A and B, respectively. The measured filter clearance of colistin is about 40% of the renal clearance observed for patients with normal renal function (at a creatinine clearance of 120 mL/min). In addition, the dialytic colistin removal efficiency could be further improved, whenever necessary, by increasing the dialysate flow and effluent volume with respect to the blood flow.

CONCLUSIONS

The 2 LC–MS/MS methods developed and validated for the quantitative determination of colistins A and B in human plasma and ultrafiltrate proved to be adequate to measure the distribution of colistin administered to critically ill patients with renal failure and subjected to CRRT. In particular, these methods were successfully applied to measure the concentration of colistins A and B in real samples from 4 patients undergoing CVVHDF.

The experimental results proved that colistin B crossed the dialytic polysulfone membrane more freely than colistin A, resulting in a more extensive elimination through the ultrafiltrate flow. The SCs experimentally measured for both colistins from ex vivo patients proved to be close to the values expected on the basis on membrane cutoff and a plasma-free colistin fraction of 34%.

The clearances for colistins A and B were sufficiently stable over the entire length of the dialysis section to produce a constant and efficient drug removal during 48 hours of the CVVHDF treatment. Even if the clearance of colistins A and B, that the polysulfone filter can produce, is limited to the exchangeable free colistin fraction, their experimental values represent a significant fraction (about 40%) of expected renal clearance in patients with normal renal function.

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Table 1

Retention times, SRM transitions, and optimal declustering potential and collision energy for the analytes and internal standards in tandem mass spectrometry experiments.

Analyte	Retention time (min)	SRM ions (m/z)	Declustering Potential (V)	Collision Energy (V)
Colistin A	5.66	585.7/101.1	42	53
		585.7/241.3		29
		585.7/202.3		31
Colistin B	5.55	578.7/101.1	43	50
		578.7/227.3		31
		578.7/202.3		33
Polymyxin B1	5.72	602.6/101.3	40	49
		602.6/202.1		33
		602.6/241.2		28
Polymyxin B2	5.61	595.6/100.9	45	53
		595.6/184.3		48
		595.6/202.5		29

Table 2

Method validation results, including intralaboratory precision, trueness, matrix effect and recovery, at low and high concentration, plus LOD (calculated and evaluated) LOQ and R^2 for the two analytes in plasma and ultrafiltrate. Matrix effect and recovery were evaluated also for the two internal standards.

	Plasma concentration (ng/mL)	UF concentration (ng/mL)		Plasma	UF
Colistin A	305.0	152.5	Within-run precision as CV% (low level)	6.31%	4.27%
	3050	1525	Within-run precision as CV% (high level)	4.28%	3.30%
	305.0	152.5	Between-run precision as CV% (low level)	5.92%	6.21%
	3050	1525	Between-run precision as CV% (high level)	6.36%	8.99%
	305.0	152.5	Within-run trueness (n =10) (low level)	85.4%	101.2%
	3050	1525	Within-run trueness (n = 10) (high level)	99.6%	102.8%
	305.0	152.5	Between-run trueness (n = 5) (low level)	98.2%	109.7%
	3050	1525	Between-run trueness (n = 5) (high level)	95.6%	103.8%
	305.0	152.5	Matrix effect (low level)	27%	31%
	3050	1525	Matrix effect (high level)	27%	22%
	305.0	152.5	Recovery (low level)	62%	68%
	3050	1525	Recovery (high level)	46%	81%
	LOD calculated (ng/mL)			18.5	19.4
	LOD evaluated (ng/mL)			45.7	30.5
	LOQ (ng/mL)			152	91.5
	R^2			0.9970	0.9978
Colistin B	618.0	309.0	Precision as CV% (low level)	4.27%	5.45%
	6180	3090	Precision as CV% (high level)	3.30%	7.11%
	618.0	309.0	Trueness (low level) (n =10)	564.5±24.8 ng/mL	305.5±16.7 ng/mL

	6180	**3090**	**Trueness (high level) (n = 10)**	6478±213 ng/mL	3000±213 ng/mL
	618.0	**309.0**	**Matrix effect (low level)**	39%	36%
	6180	**3090**	**Matrix effect (high level)**	31%	32%
	618.0	**309.0**	**Recovery (low level)**	53%	66%
	6180	**3090**	**Recovery (high level)**	51%	66%
	LOD calculated (ng/mL)			34.0	33.9
	LOD evaluated (ng/mL)			92.0	61.8
	LOQ (ng/mL)			306	185
	R^2			0.9987	0.9995
Polymyxin B1	**849**	**1359**	**Matrix effect**	30%	34%
	849	**1359**	**Recovery**	50%	84%
Polymyxin B2	**225**	**361**	**Matrix effect**	32%	33%
	225	**361**	**Recovery**	47%	76%

Table 3

Demographic and clinical data of patients who received CMS and CVVHDF therapy.

Patients (n°)	Age (years)	Sex	Diagnosis	Colistin (Sample day)	NA/DA[a] (μg/Kg/min)	Infection (Hemoculture)	Exitus
1	58	M	Burns 60%	5	0.60/3.0	Acinetobacter Baumanni	YES
2	60	M	Ulceration in diabetes	14	0.30/3.0	Acinetobacter Baumanni	NO
3	89	M	Burns 40%	3	0.50/9.0	Acinetobacter Baumanni	YES
4	62	M	Pneumonia	3	0.00/8.0	Klebsiella Pneumoniae	NO

[a] NA = noradrenaline; DA = dopamine

Table 4

Blood (pre- and post-filter) and ultrafiltrate colistin A and B concentrations in real samples for four serial critically ill patients undergoing CVVHDF.

Patient	Collection time	[Colistin A] (μg/mL)			[Colistin B] (μg/mL)		
		Pre-filter	Post-filter	Ultrafiltrate	Pre-filter	Post-filter	Ultrafiltrate
1	10 min	3.75	3.07	1.16	1.63	1.56	0.78
	3 h	4.39	3.78	1.34	1.88	1.80	1.10
	24 h	5.25	4.57	2.06	2.24	2.05	1.34
2	3 h	5.39	4.26	1.32	2.23	1.80	0.89
	24 h	8.80	8.81	2.16	4.50	3.33	1.39
	48 h	9.47	8.08	2.08	3.29	2.78	0.97
3	10 min	5.00	4.21	2.30	1.78	1.10	0.68
	3 h	5.31	4.88	2.00	1.75	1.65	0.75
	24 h	5.04	5.43	2.25	1.42	1.45	0.73
	24 h (+1 day)	6.54	5.49	1.97	2.10	1.68	0.71
	24 h (+5 day)	5.75	4.12	2.24	1.89	1.13	0.60
	24 h (+8 day)	4.96	4.71	1.84	1.73	1.26	0.57
4	3 h	1.61	1.54	0.73	0.32	0.29	0.27
	6 h	2.06	2.34	0.83	0.43	0.49	0.26
	12 h	1.90	2.00	0.69	0.44	0.46	0.22
	24 h	1.35	1.54	0.32	0.25	0.31	0.15

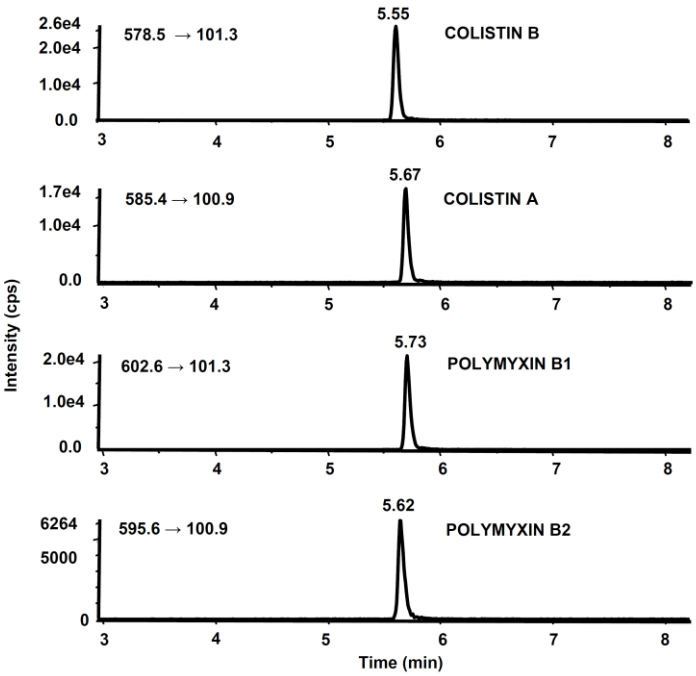

FIGURE 1. A, SRM chromatographic profiles for blank plasma; B, Blank plasma spiked at LOQ levels for both colistins; C, Pre-filter plasma of patient 1 collected 10 minutes after the beginning of the CVVHDF session; D, Blank UF; E, blank UF spiked at LOQ levels for both colistins; and F, UF of patient 1 collected 10 minutes after the beginning of the CVVHDF session.

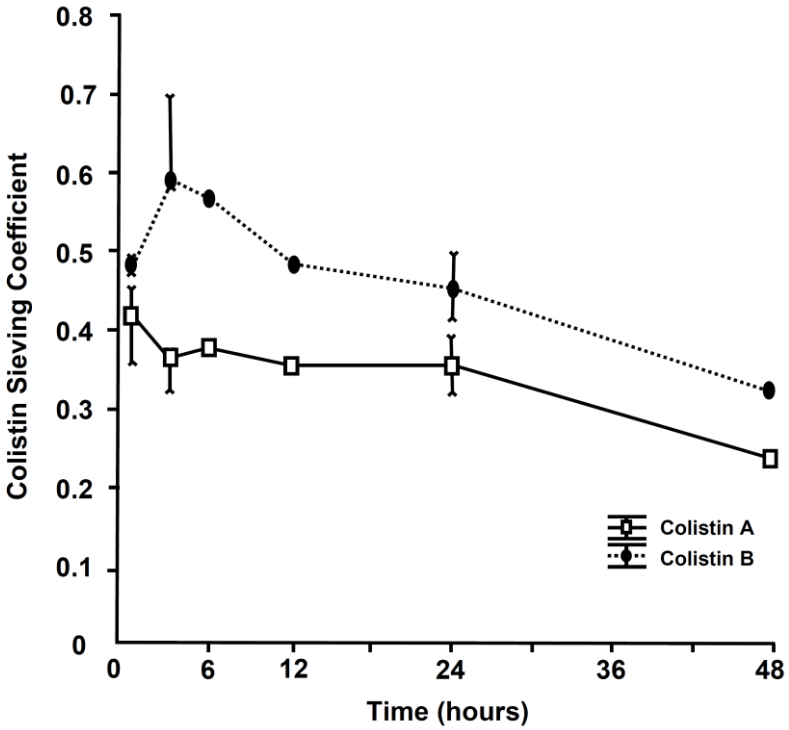

FIGURE 2. SC for colistins A and B during 48-hour CVVHDF sessions.

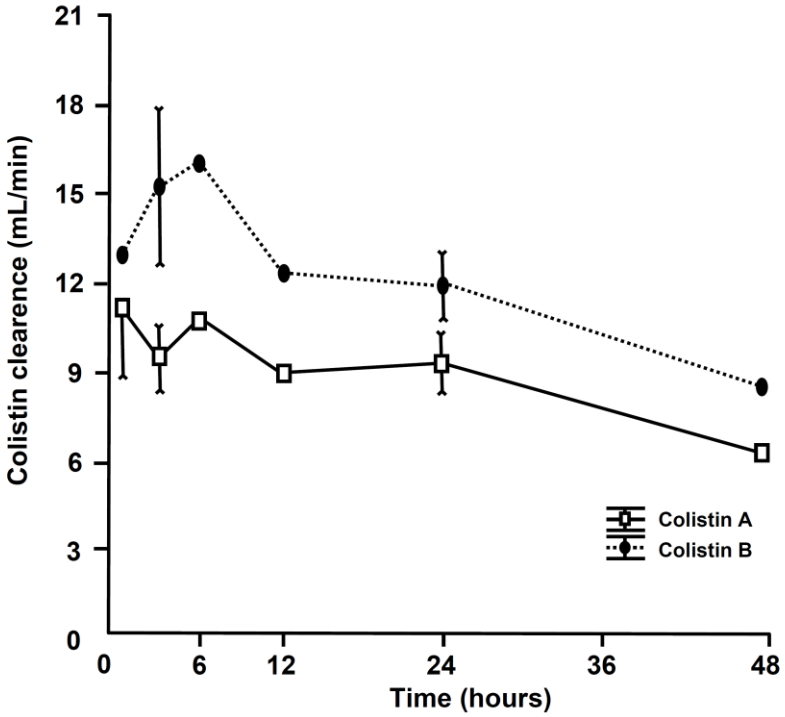

FIGURE 3. Extracorporeal clearance of colistins A and B during 48-hour CVVHDF sessions.